

Pink Eye Is an Unusual Periderm Disorder Characterized by Aberrant Suberization: A Cytological Analysis

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ABSTRACT

Potato tuber pink eye (PE) is a disorder of unknown origin that results in significant postharvest quality deterioration and rot. Little is known about the physiology of PE, including the characteristic tissue autofluorescence that defines the PE syndrome. The objective of this research was to identify the source of PE-induced autofluorescence and PE-related susceptibility to infection. The suberized barrier of the native periderm and cellular characteristics of neighboring parenchyma tissues were investigated to determine their involvement in the PE disorder. The results create a new physiological model describing the disorder and addressing the enigma of PE. Characteristics of the PE model emerge from the following results: (1) the integrity of the suberized barrier of the native periderm was compromised or absent in some surface areas of PE tubers thereby implicating the breakdown of the native periderm and its associated suberin barrier with PE and the susceptibility of PE tubers to pathogen infection; (2) the PE complex was characterized by unusual suberin poly(phenolic) (SPP) accumulations in the cortical parenchyma followed by latent suberin poly(aliphatic) (SPA) accumulations that were generally insufficient to form a complete barrier that was competent to block infections by pathogenic bacteria and fungi; (3) the aberrant absence or compromised integrity of the suberin barrier, including associated waxes, resulted in erratic increased susceptibility to water vapor loss known to cause tuber

shrinkage and flaccidity; (4) widespread accumulations of SPP on parenchyma cell walls were the durable source of autofluorescence commonly used to determine the presence of the disorder; (5) the erratic development of unusual internal phellogen and periderm layers that, if complete with SPA, blocked hyphal advancement; (6) combined, the data provide a plausible explanation for PE infection court and rot anomalies as they occur without ingress of a wound opening. Results also demonstrated that neutral red may be used as a sensitive fluorochrome to detect intact hydrophobic areas in hyphae. Collectively, the results provide compelling evidence that the PE disorder includes a physiological basis.

RESUMEN

El ojo rosado (PE) del tubérculo de papa es un desorden de origen desconocido que deviene en un significativo deterioro de la calidad de poscosecha y pudrición. Muy poco se conoce a cerca de la fisiología de este desorden incluyendo la auto fluorescencia del tejido que caracteriza al síndrome de PE. El objetivo de esta investigación fue identificar la fuente de auto fluorescencia inducida por PE y la susceptibilidad a la infección. Se investigaron, la barrera suberizada del peridermo nativo y las características celulares del tejido del parénquima vecino, para determinar su participación en el

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ABBREVIATIONS: ELISA, enzyme-linked immunosorbant assay; PE, pink eye; SE, standard error; SPP, suberin poly(phenolic(s)); SPA suberin poly(aliphatic(s)); PDA, potato dextrose agar; FAA, formalin-acetic acid-alcohol

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desorden que produce el PE. Los resultados han creado un nuevo modelo fisiológico que describe el desorden y que señala el enigma de PE. Las características del modelo emergen de los siguientes resultados: (1) la integridad de la barrera suberizada del peridermo nativo estuvo comprometida o ausente en algunas áreas de la superficie de tubérculos afectados implicando una descomposición del peridermo nativo, la integridad de la barrera de suberina asociada, y la susceptibilidad de los tubérculos a la infección por patógenos, (2) el PE estuvo caracterizado por una extraordinaria acumulación de suberina polifenólica (SPF) en el parénquima, seguida por una latente acumulación de suberina polialifática (SPA) que fueron generalmente insuficientes para formar una barrera completa capaz de bloquear infecciones de bacterias y hongos patógenos, (3) la ausencia aberrante o compromiso de la integridad de la barrera de suberina, incluyendo ceras, dio como resultado un aumento errático de la susceptibilidad a la pérdida de vapor de agua, causas conocidas de la reducción y flacidez del tubérculo, (4) la acumulación generalizada de SPF en las paredes de las células del parénquima fue el origen constante de la autofluorescencia, utilizada comúnmente para determinar la presencia del desorden, (5) el desarrollo errático de capas de felógeno interno inusual y capas de peridermo, las cuales cuando se complementan con SPA, bloquean el avance de hifas, (6) combinados los datos, proporcionan una explicación plausible para el ingreso de PE y anomalías de la raíz, como ocurre sin la abertura de una herida de ingreso. Los resultados también han demostrado que se puede utilizar el rojo neutro como un fluorocromo sensible para detectar las áreas hidrofóbicas en hifas. En conjunto, los resultados proporcionan una evidencia precisa de que el PE incluye una base fisiológica.

INTRODUCTION

Pink eye (PE) is a serious tuber disorder of unknown origin that occurs in major potato-growing regions of North America (Secor and Gudmestad 2001; Nolte et al. 1993). PE can be found in carefully grown seed, processing, and fresh market potatoes. PE has been known to cause extensive quality and rot problems prior to harvest and during storage. Despite the costly losses that result from PE, little is known

about the cellular characteristics and the biology that may be used to determine the cause of this disorder.

Pink eye is characterized by an ephemeral pink coloration frequently located around the eyes and various parts of the surface of freshly harvested tubers, particularly at the bud-end. Equally characteristic is a durably strong autofluorescence directly beneath the surface of the affected areas of the tuber and a high incidence of rot (Nolte et al. 1993). The source of this strong autofluorescence has not been unequivocally determined, even though it is diagnostic for the disorder. Chlorogenic acid, esculin, and scopoletin have been shown to be associated with PE and pathogen invasions (Nolte et al. 1993). A layer of tissue directly beneath the surface of PE areas is generally browned, indicating oxidation and possibly cell death. In addition, PE tubers frequently develop a corky-like thickened skin (corky patch or bull hide), which complicates steam peeling and results in processing defects (Nolte et al. 1993).

Despite its deleterious impact on the potato industry, the cause of PE is not known. With the exception of research by Nolte et al. (1993), little has been done to determine the biochemical and physiological characteristics that initiate, accompany, and/or control the PE disorder. Attempts to identify a causal organism for PE have been unsuccessful due to the sporadic appearance of this disorder and the inability to complete Koch's postulates for any pathogen or combination of pathogenic organisms. Consequently, the development of approaches to prevent or reduce the occurrence and severity of PE has been severely hampered.

To date, efforts to reproduce the disorder under controlled conditions have been unsuccessful. No greenhouse or growth-chamber conditions for inducing PE have been reported. The only means of analyzing and determining critical properties of the disorder is through donated samples from cooperating growers whose fields are exhibiting PE tubers.

Various environmental conditions are known to be associated with PE development, including excess water late in the season in conjunction with higher than normal air and soil temperatures and premature vine death that reduces canopy cover (Goth et al. 1993; Secor and Gudmestad 2001). Based on field observations, it appears that soil temperatures that exceed optimums for tuber bulking and soil moisture content at, or exceeding, saturation predisposes potato tubers to development of PE (Gudmestad, pers comm). Poor canopy health due to factors such as early dying can limit soil water

transpiration and contribute to the development of PE. Previously, *Pseudomonas fluorescens* (Trevisan) Migula, *Verticillium* spp. (early dying), and *Rhizoctonia solani* Kuhn were thought to be linked with the occurrence of PE and these environmental conditions (Goth et al. 1993, 1994; Secor and Gudmestad 2001). However, these pathogens have not been proven to be required for PE and while the combined environmental conditions noted above are conducive to PE they do not consistently lead to PE development.

The lack of information on the biology of PE has been an impediment in developing strategies to address this disorder. Information is lacking on the cellular characteristics of surface and neighboring internal tuber tissues, the source of the intense autofluorescence that is diagnostic for PE, and the integrity of the native periderm and its suberized cells. It is known, however, that the suberized barrier of the periderm is of crucial importance because it provides broad durable resistance to infection (Lulai 2001). Lulai and Corsini (1998) showed that (1) suberin poly(phenolics) (SPP) accumulate first on suberizing cell walls and provide resistance to bacterial but not fungal infection; (2) suberin poly(aliphatics) (SPA) accumulate next and are glycerol crosslinked (Graca and Pereira 2000) to the SPP; and (3) resistance to fungal infection is present only after the SPA barrier is in place. Cinnamic acid derivatives form the SPP barrier while certain fatty acids form the SPA barrier (Bernards 2002). Soluble waxes embedded into the suberin biopolymer are essential in providing resistance to water vapor loss, which results in shrinkage during potato storage (Soliday et al. 1979; Lulai and Orr 1994, 1995; Schreiber et al. 2005). During tuber wound-healing, a wound-induced closing layer is formed, i.e., one or two layers of existing cells suberize at the wound surface as described above. As the closing layer is formed, and if favorable conditions exist, an adjoining wound-periderm may form under the closing layer via development of a phellogen (a meristematic layer of cells). The phellogen produces files of phellem cells (newly formed suberized cells) and an underlying layer of phelloderm cells (Lulai 2001). Unfavorable conditions may allow accumulations of suberin biopolymers on existing cell walls, i.e., formation of a closing layer, but preclude development of the layer of phellogen cells required to produce a wound-periderm. It is likely that these elements of the suberin model and suberization are pertinent to the syndrome created by the PE disorder. The above knowledge gaps were investigated and a new physiological model developed to describe PE.

MATERIALS AND METHODS

More than 12 tuber samples from russeted and white-skinned genotypes ('Russet Burbank', 'Shepody', FL1879) afflicted with PE were obtained and analyzed from a range of growing areas within the U.S.A. (including ND, ID, MN, MI, WI, NE) during 1999 through 2005. All analyses were conducted two or more times in triplicate. For conciseness and consistency, all results presented, with the exception of Figure 1, were obtained from the cultivar Russet Burbank. Unless indicated otherwise, the results are consistent with those obtained from other cultivars and samples.

Pink Eye Screening and Identification

Tubers obtained directly upon harvest were visually screened for the ephemeral light pink coloration about the buds and elsewhere on the tuber surface as a possible indication of PE. The definitive presence of PE in freshly harvested and stored tubers was determined by removing surface tissues through successive abrasions or through the use of a specially fabricated slicer designed to remove 0.75-mm-thick sections of tissue. The freshly exposed cortical parenchyma cells were viewed in the dark under a dual 15 watt Sylvania T8 Blacklight Blue ultraviolet fluorescent lamp system (wavelength ~ 300 nm). Tubers afflicted with PE fluoresced with a brilliant blue color (Nolte et al. 1993). Similar tissue areas from non-PE tubers did not fluoresce. The source of this strong autofluorescence was identified by microscopically analyzing cells located at and near the surface of PE tubers and comparing the results with that of healthy/non-PE tubers.

Suberin Poly(phenolic) and Poly(aliphatic) Determination

Tissue blocks (~1 cm wide × 0.5 cm deep × 1.0 cm long) were excised and fixed in a solution of formalin:acetic acid:95% ethanol: water (3:1:10:7, v/v/v/v: FAA). PE tissue blocks were taken from areas directly neighboring those identified using the blacklight blue fluorescent lamp system. Tissues were hand sectioned and microscopically analyzed as previously described using autofluorescence and berberin/rheuthinium red to detect the poly(phenolic) component of suberin (Lulai and Corsini 1998). Results obtained using berberin/rheuthinium red and autofluorescence were similar; consequently autofluorescence was routinely used on most of the samples. The cytofluorochrome neutral red and the blue

violet autofluorescence quenching agent toluidine blue O were used in combination to probe for the presence of the poly(aliphatic) component of suberin as previously described with the exception of the incubation period (Lulai and Morgan 1992; Lulai and Corsini 1998). These hand-sectioned tissues were incubated overnight in the toluidine blue O solution, then rinsed in water, and incubated for 4 h in the neutral red solution. Microscopy was performed with a Zeiss Axioskop 50 microscope configured for epifluorescent illumination as previously described (Lulai and Corsini 1998). Digital images were obtained with a Zeiss color AxioCam camera. (Carl Zeiss Inc., Thornwood, NY).

Visualizing Fungal Hyphae

Fungal hyphae and associated tuber parenchyma cell material were identified by staining with trypan blue (Sneh et al. 1994). Sections of PE tuber tissue and cultured *Rhizoctonia solani* hyphae were incubated overnight in acidified (HCl) 0.5% trypan blue in lactophenol. Tissue sections and hyphae were microscopically analyzed using white light, and digital images were obtained as outlined above. The combination of neutral red and toluidine blue O, described above, was also found useful in identifying vegetative hyphae through the partitioning of neutral red into hydrophobic areas (vacuoles/lipid bodies) (Gahan 1984; Weber 2002). *Rhizoctonia solani* obtained from symptomatic potato tubers and sugar beet were cultured on potato dextrose agar in the dark at 24 C. Vegetative hyphae scraped from plates were stained with trypan blue, suspended in water, and mounted on slides for light microscopy as described above.

Immunodetection of *Rhizoctonia solani*

ELISA for detection of *R. solani* was conducted on healthy tuber samples not afflicted with PE, PE samples with hyphae, and PE samples without hyphae. These samples were taken from the pool of FAA-fixed tissue described above. A pure culture of *R. solani* obtained from black sclerotia on infected tubers was maintained on potato dextrose agar (PDA) and grown at 24 C. Tissue sections of approximately the same size were cut from each sample block and rinsed three times in distilled water to remove the FAA. The sections were then homogenized in distilled water (1 g mL⁻¹) and the resulting suspension autoclaved for 12 min to destroy endogenous phosphatase activity. Cultured *R. solani* that was scraped from the surface of PDA and homogenized in distilled water (1 g 10 mL⁻¹),

and healthy tuber tissue from potato clone FL 1879, not fixed in FAA, but prepared and autoclaved as above, served as internal experimental controls.

Aliquots (75 µL) of the above extracts were mixed with 100 µL of coating buffer (0.16% Na₂CO₃, 0.29 % NaHCO₃, pH 9.6) in the wells of an immunological assay plate and incubated for 18 h at 4 C. The wells of the plate subsequently were rinsed five times with phosphate-buffered saline pH 7.4 containing 0.05% Tween-20 (PBST; 200 µL per well). After removal of the last wash, each well received 100 µL of ELISA Sample Buffer (PBST containing 2% polyvinylpyrrolidone-40 and 0.2% ovalbumin) containing a 1:200 dilution of rabbit anti-*R. solani* IgG (Loewe Diagnostics, Germany) and incubated for 5 h at 22 C. Following five washes with PBST, each well received 100 µL of ELISA Sample Buffer containing a 1:1,000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich, St Louis, MO). The plate was incubated at 22 C for 1 h followed by washing of the wells five times with PBST. After removal of the last wash, each well received 200 µL of substrate buffer (9.8% diethanolamine pH 9.6) containing 0.8 mg of *p*-nitrophenyl phosphate. The plate was incubated at 37 C for 2 h and was then scanned at 405 nm in a Tecan plate reader. Two separate samples for each treatment were used in the test and each sample was tested in duplicate on the ELISA plate.

Determination of Tuber Water Vapor Conductance

Water vapor conductance was measured through healthy native periderm of tubers not afflicted with PE (control) and through the putative periderm of PE afflicted tubers. Three measurements were taken from each of 10 tubers for each sample. Vapor conductance measurements were determined with a LiCor model LI-1600M Steady State Porometer (LI-COR, Inc., Lincoln, NE) as previously described (Lulai and Orr 1994, 1995). Porometric measurements were corrected for boundary layer resistance as indicated by the manufacturer.

RESULTS

Results shown are representative of those obtained from samples derived from different field locations, states, and crop years (1999-2005). The severity of the PE symptoms was variable from tuber to tuber as is characteristic of the disorder.

Identifying PE Tubers

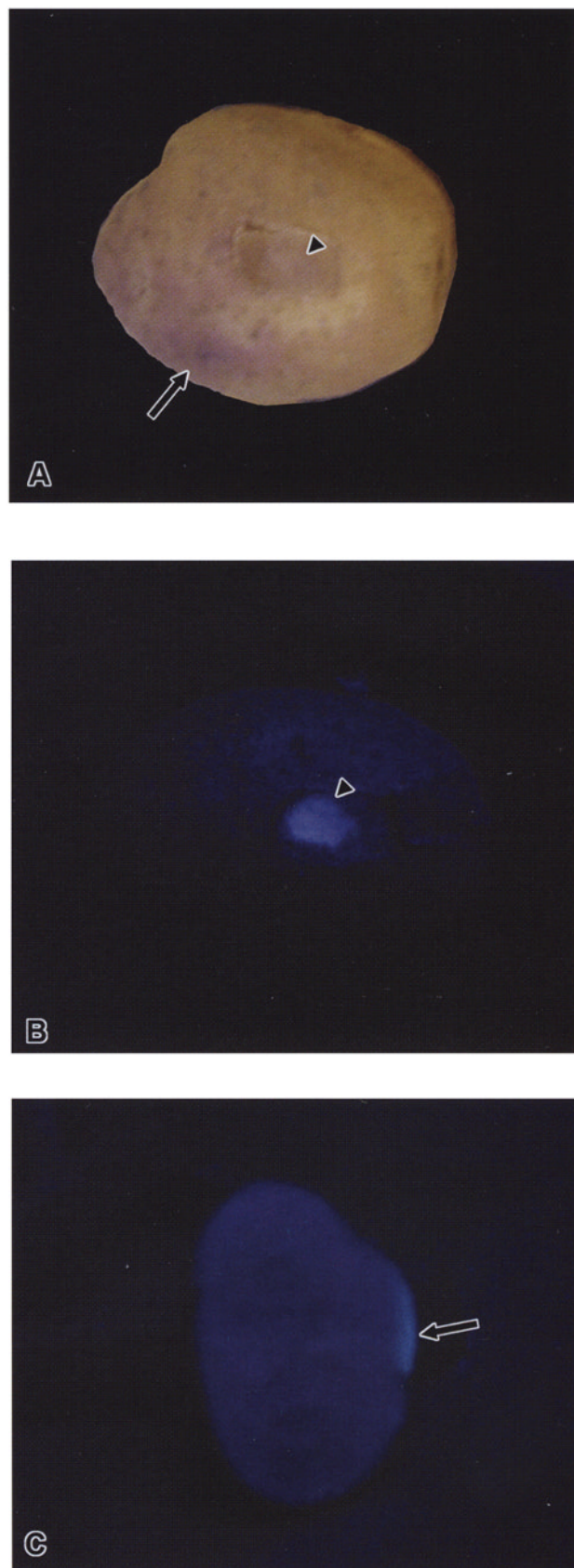
Tubers afflicted with PE often expressed the partially symptomatic light pink color associated with the eyes of the tuber. These discolorations, often used to screen for PE tubers directly from the field, were generally located around the eyes of the tuber at the bud end and elsewhere on the tuber surface as the disorder became more pronounced (Figure 1A). However, the pink colorations were generally faint and always ephemeral, lasting from several hours to a few days after harvest. The characteristic strong autofluorescence, located directly beneath the surface of the tuber (Figure 1B), was the primary means of identifying and verifying the presence of the PE disorder in potato tuber. The PE autofluorescence was localized to the specific areas afflicted by the PE disorder and was not uniformly expressed across the tuber subsurface, i.e., cortical parenchyma and/or parenchyma (Figure 1C).

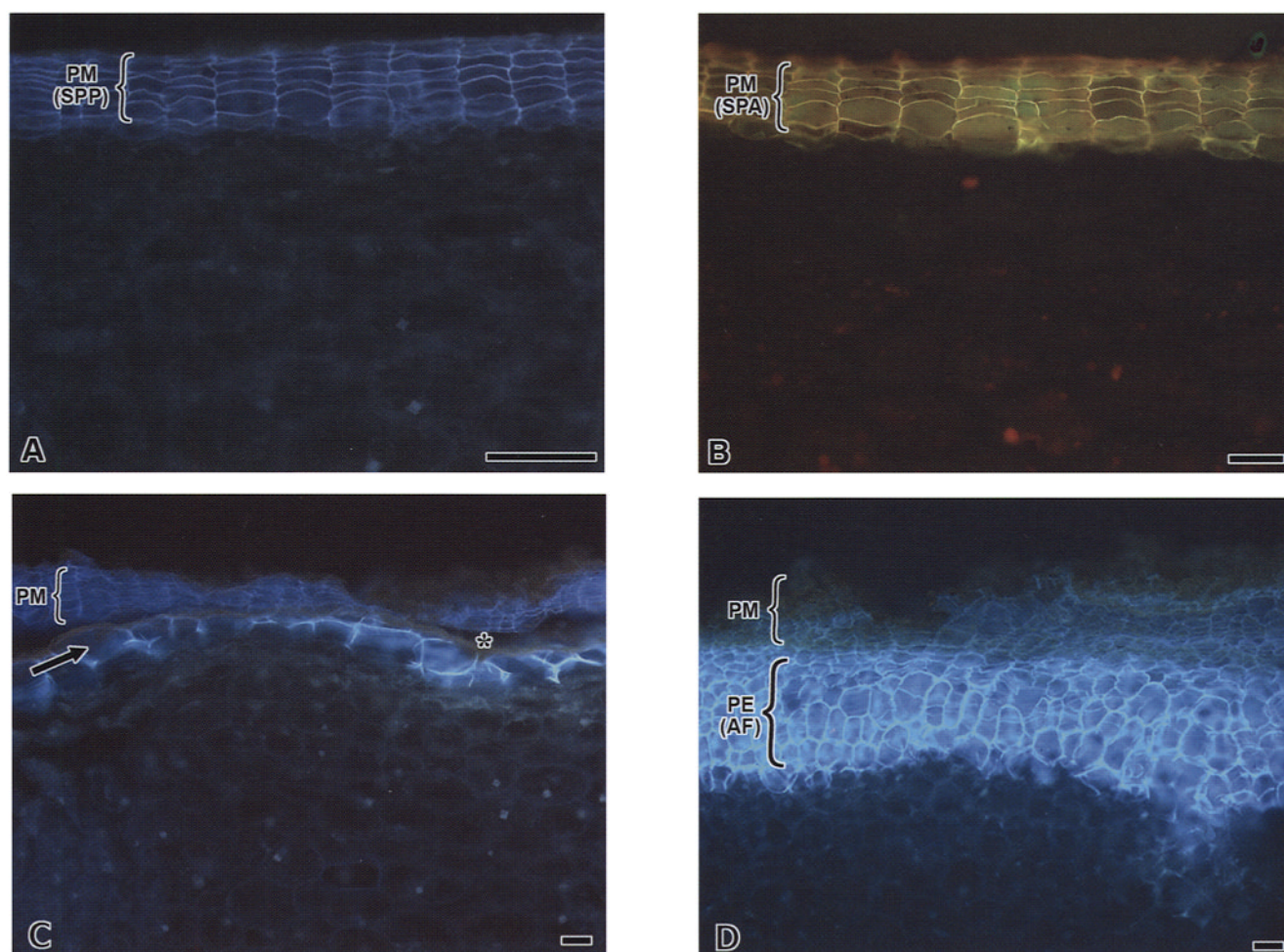
Cellular Characteristics of Healthy Tuber Periderm and Cortical Parenchyma

Autofluorescence of phellem and closely associated cells differed between healthy and PE tubers when analyzed microscopically. Phellem cells in healthy native periderm autofluoresced due to the presence of SPP on the cell walls (Figure 2A). SPA, which accumulate after the SPP on phellem cell walls, yielded a strong specific fluorescent signal only when treated with the fluorochrome neutral red and the non-specific autofluorescence was quenched with toluidine blue O (Figure 2B). Fluorescence of the suberin biopolymers located on the phellem cell walls reveals the well-organized files of cells created by the meristematic action of the phellogen as the periderm formed. Phellogen, phelloderm, and cortical parenchyma cells located directly beneath the phellem do not fluoresce in healthy, normal tuber tissues. Starch granules beneath the periderm in the cortical parenchyma were also often visible under both UV-illumination and with neutral red treatment in both healthy and PE tubers.

FIGURE 1.

Tuber (Shepody) PE characteristics under (A) white light and under (B and C) UV (Blacklight Blue fluorescent) lighting. (A) Tuber showing surface ephemeral pink coloration (arrow) and area of tuber skin abraded (arrowhead) for determination of UV autofluorescence in underlying cortical tissue. (B) UV autofluorescence of PE cortical parenchyma tissue exposed after abrasion of surface tissue as shown in (A) (arrowhead). (C) Tuber cut in half exposing PE autofluorescent area (arrow) and remaining tuber areas that do not fluoresce.



**FIGURE 2.**

Native periderm and underlying cortical parenchyma cells from: (A and B) healthy tubers not afflicted with PE, and (C and D) PE tuber tissue areas viewed by epifluorescent microscopy. In healthy tubers: (A) suberin poly(phenolics) (SPP) located on periderm phellem (PM) cell walls autofluoresce under ultraviolet excitation while underlying cortical parenchyma cell walls are barely visible; (B) suberin poly(aliphatics) (SPA) located on phellem (PM) cell walls specifically fluoresce under blue violet excitation after treatment with neutral red and toluidine blue O, while cortical parenchyma cell walls are devoid of the biopolymer. The phellogen and phelloderm layers, directly beneath the PM, are not discernable under these conditions. In PE tubers: (C) phellem cells of native periderm are breaking away from the underlying tissue at the phellogen cell layer (*), and neighboring phelloderm and parenchyma cells are beginning to form an internal “closing layer” (arrow) with the accumulation of autofluorescent SPP on these existing cell walls—note the resulting darkened layer at the fractured phellogen; (D) extensive PE development induced accumulation of autofluorescent (AF) SPP on cortical parenchyma cell walls—note that phellem cells of native periderm are present, but in this case feature reduced autofluorescence indicating deterioration of the suberin barrier. Bar = 100 μm .

PE Tuber Autofluorescence and Cellular Characteristics

Microscopical analyses of tuber periderm and adjoining cortical parenchyma from PE-afflicted tubers clearly illustrate the accumulation of autofluorescent compounds on cell walls directly beneath the surface (Figures 2C, 2D, 3, and 4). The development of autofluorescence on parenchyma cell walls may occur minimally in some areas of the tuber (Figure 2C) and in greater amounts elsewhere (Figure 2D).

Autofluorescence in the PE-afflicted tissues illustrated in Figure 3A clearly reveal the initiation of a meristematic layer well beneath the tuber surface and below the PE-induced fluorescence associated with the cortical parenchyma cells. The meristematic layer is indicated by the development of files of rectangular phellem cells amidst the circular to oblate shaped cells of the parenchyma. There is little or no accumulation of autofluorescent material below the internal phellem cells originating from the meristematic layer induced by the PE disorder.

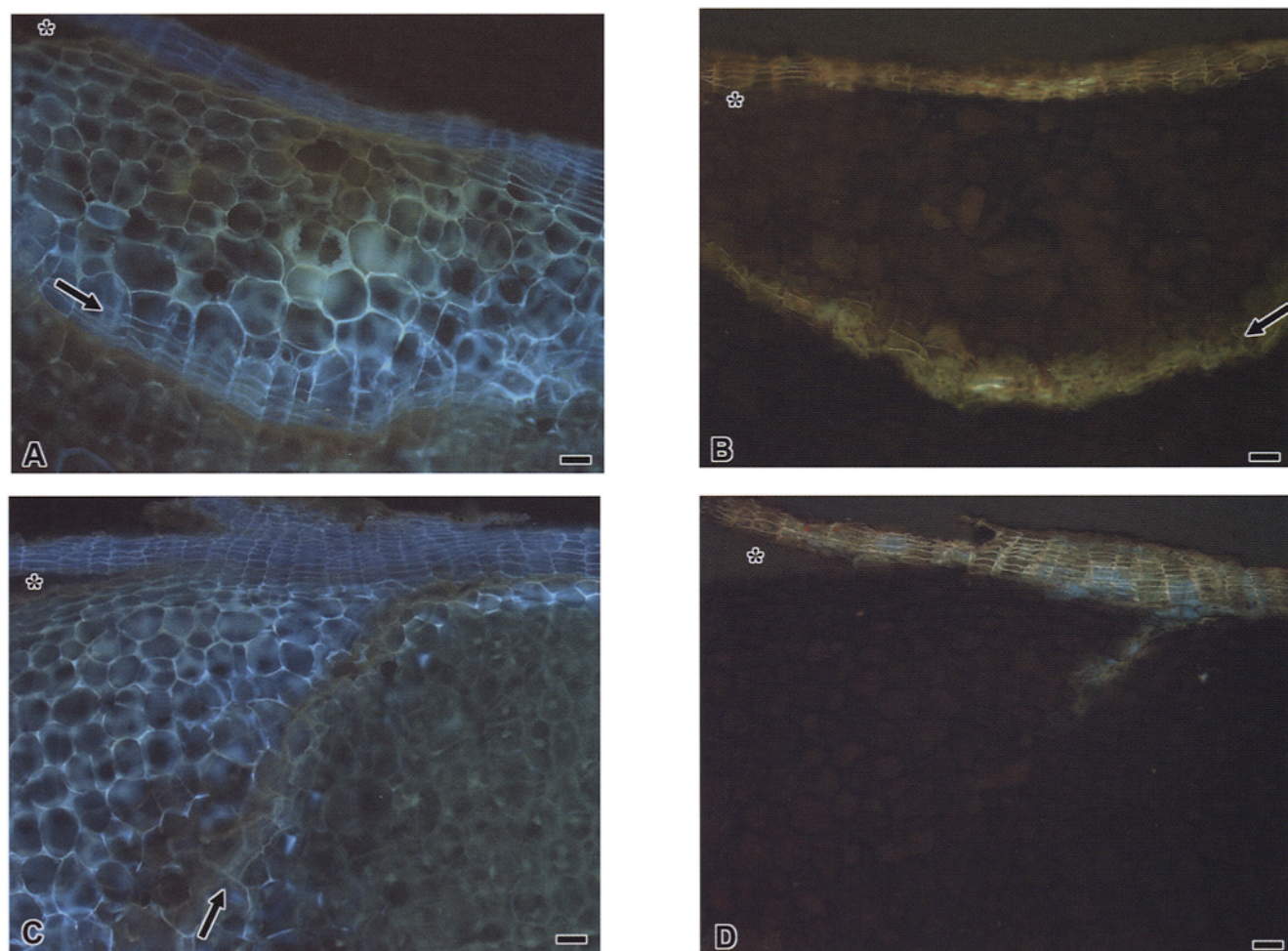


FIGURE 3. PE tissues viewed under (A and C) ultraviolet excitation causing autofluorescence of SPP and under (B and D) blue violet excitation after treatment with neutral red/toluidine blue O causing specific fluorescence of SPA. Parallel sections (A and B) show some separation of the native phellem from the tuber (*) and the development of a phellogen producing organized files of suberized cells (arrow) deep within the cortical parenchyma. Parallel sections (C and D) illustrate separation of the native phellem from the tuber (*) and PE-induced changes. (C) Extensive accumulations of autofluorescing SPP on cortical parenchyma cell walls, but without corresponding (D) accumulation of SPA. Note (C) the diagonal development of a nascent phellogen (arrow), but with a neighboring darkened layer suggesting deterioration and without phellem cells and associated SPP fluorescence. Bar = 100 μ m.

der (Figure 3A). As with the development of surface phellem cells found in healthy native periderm, the internal phellem cells also accumulated SPA as illustrated in Figure 3B. In some cases, the meristematic layer appeared to be nascent, with evidence of the internal phellogen barely detectable, and not sufficiently developed to create files of internal phellem cells (Figure 3C). There was little or no accumulation of SPA along the line formed by the nascent meristematic zone (Figure 3D). However, the meristematic layer again appeared to delimit a zone below which further accumulation of fluorescent SPP was reduced or terminated.

The amount and location of PE-induced accumulation of SPP and SPA on cortical parenchyma cell walls was varied as illustrated by Figures 2C, 2D, 3, 4, and 5. The PE-induced accumulation of the suberin polymers could develop directly under the phellem of the native periderm and then extend deeper into the tissue (Figure 4). In some cases, the autofluorescence developed several cell layers away from the phellem. Areas of PE tissues frequently lacked the protective barrier provided by the phellem cells of the native periderm (Figure 4A and 4B). Similar morphology was found, with varying severity, throughout the PE samples. In many cases the native phellem began to

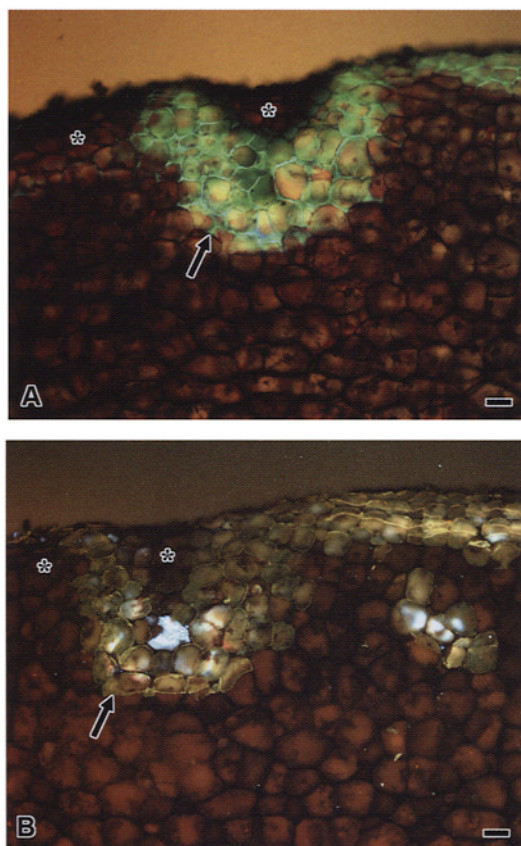


FIGURE 4. Parallel sections of PE tissues viewed under (A) ultraviolet excitation and white light after treatment with berberine rheuthinium red resulting in specific fluorescence of SPP (arrow) and under (B) blue violet light after treatment with neutral red/toluidine blue O to specifically cause fluorescence of SPA (arrow). Note that some surface areas are without the protection of a suberized barrier provided by the phellem (*). Bar = 100 μ m.

TABLE 1—The effect of the pink eye disorder on tuber water vapor conductances.

Tuber area measured*	Porometric Vapor Conductance ($\text{mMol M}^{-2} \text{S}^{-2}$)		
	Mean**	Range	(SE)
Sample 1			
Control (No PE)	35.5	21.3 to 62.5	(6.4)
PE areas	153.3	65.6 to 1038.5	(210.9)
Sample 2			
Control (No PE)	11.9	0.6 to 43.0	(12.8)
PE areas	62.3	3.2 to 273.1	(60.3)

*Sample 1 = Russet Burbank tubers obtained from 2005 Minnesota production, measured 6 d after harvest. Sample 2 = Russet Burbank tubers obtained from 2005 Idaho production, measured 14 d after harvest.

**n = 10 tubers, three measurements were taken from each tuber.

separate from the tuber at the phellogen as can be noted near the edges of Figures 2C, 2D, and 3. Existing cells that accumulated autofluorescent materials, poly(phenolics), on their cell walls were subsequently capable of accumulating SPA (Figure 4B) to complete the suberization process. However, the completion of PE-induced suberization on existing cortical parenchyma cell walls was not typical as is illustrated in Figure 3.

Tuber Water Vapor Conductance

Water vapor conductance measured through the native periderm of healthy tubers compared to that through the PE surfaces of afflicted tubers was starkly different (Table 1). The mean vapor conductance measured in different samples shortly after harvest (6 d and 14 d) was much larger for PE tuber surfaces (from four- to six-fold) compared to measurements obtained through the periderm of control tubers that did not have PE. The range and resulting SE for PE tubers was several fold greater than that of the controls that did not have PE for both the 6 d and 14 d determinations; these data indicate large variations in water vapor losses for PE tuber surfaces (Table 1). Similar results were obtained with other samples (data not shown). These wide variations in vapor conductance measurements were consistent with the wide variations in PE periderm and PE suberization aberrations illustrated in Figures 2C, 2D, 3, and 4.

Detection of Hyphae in Some PE Tubers

Figure 5 illustrates parenchyma cells in which fungal hyphae have proliferated, but without incipient rot. Hyphae were contrasted from the tuber cells by toluidine blue O/neutral red treatment (Figure 5A). Numerous hyphae were highlighted by the neutral red treatment, which partitioned into small hydrophobic areas, i.e., the vacuole or other lipid rich bodies, and fluoresced (Figure 5B). Many hyphae apparently were no longer viable and consequently did not demonstrate fluorescence from an intact vacuole or hydrophobic area and were visible only as dark strands (Figure 5B and 5C). In some areas, the neutral red treatment revealed a high density of hyphae with internal fluorescent areas while neighboring areas closer to the surface hosted non-viable hyphae with no fluorescence. The occasional development of an interior periderm and accumulated SPA are illustrated in Figure 5C. The hyphae were present in cells above the suberized barrier of the internal periderm. Cells below the internal periderm were largely devoid of hyphae.

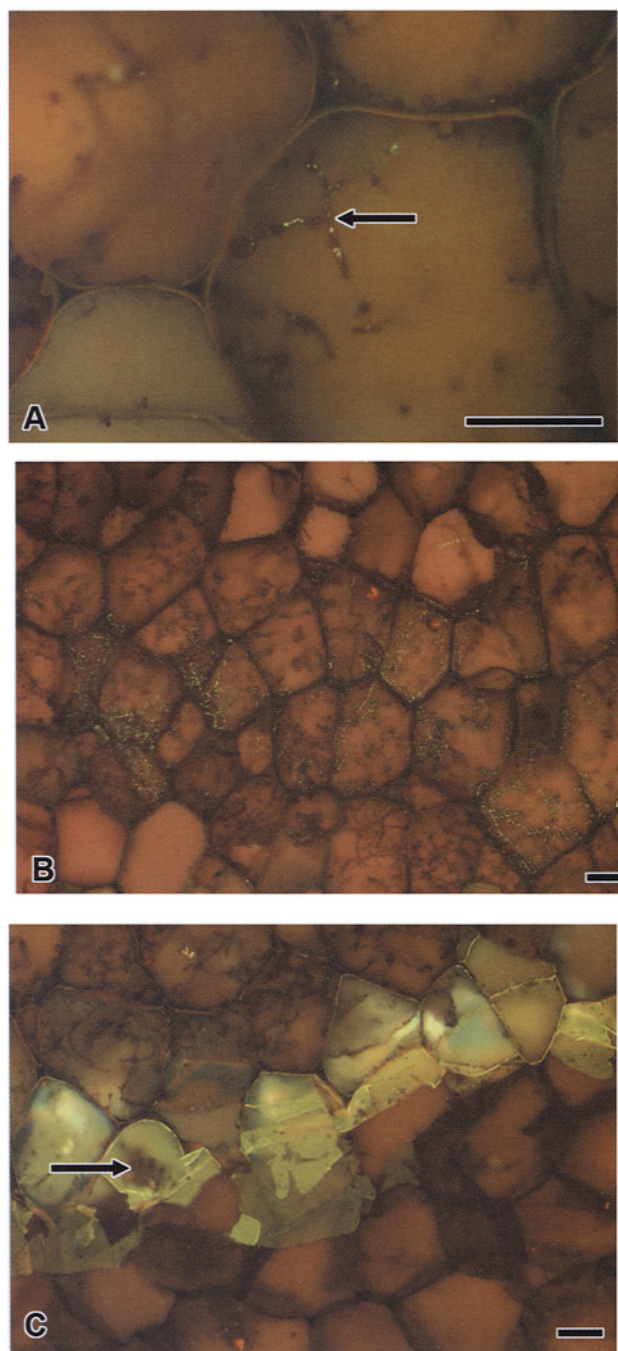


FIGURE 5.

PE-afflicted cortical parenchyma tissues viewed under blue violet excitation after treatment with neutral red and toluidine blue O to cause specific fluorescence of SPA, but also revealing the presence of fungal hyphae. Hyphae are visible as dark strands. (A and B) Hydrophobic areas of viable fungal hyphae were induced to fluoresce after neutral red partitioning. (C) Fungal hyphae are visible residing above a PE-related internal periderm, but are largely blocked from deeper penetration by the SPA barrier (arrow) of the periderm. Bar = 100 μ m

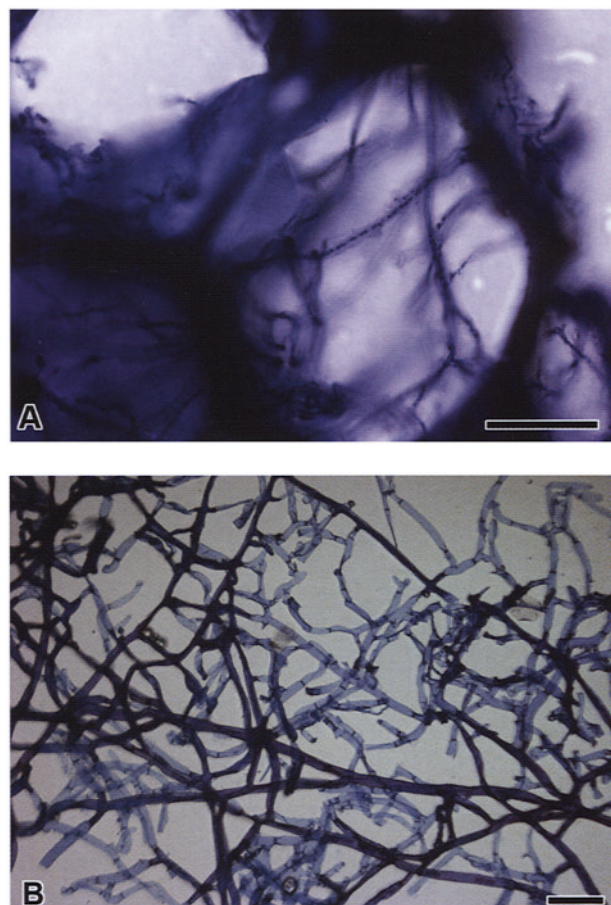


FIGURE 6.

Trypan blue stained hyphae found in PE tuber tissue and fungal cultures of *R. solani*. Right angle hyphal branching, construction of hyphae at branch nodes, and apparent multi-nucleate cells in hyphae within PE-afflicted tissue (A) are consistent with published and observed structures of cultured *R. solani* (B) Hyphal diameters determined from micrographs averaged 9 μ m and ranged from 7 to 11 μ m for the *R. solani* cultured *in vitro*, and averaged 4 μ m and ranging from 3 to 6 μ m for the hyphae grown *in vivo* in the tuber cells. Bar = 100 μ m.

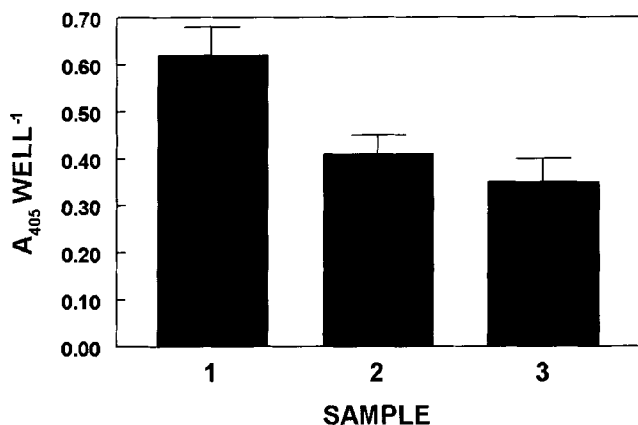


FIGURE 7.

Immunodetection of *R. solani* in PE tuber tissues. Rabbit anti-*R. solani* IgG antibody was used via an ELISA to evaluate for the presence of *R. solani*. Extracts from PE tissue that contained (1) or lacked (2) visible hyphae by microscopy were compared to healthy Russet Burbank (3) or FL1879 tissues that were putatively free of *R. solani*. Note that the PE tuber tissue containing hyphae yielded an absorbance about 33% greater than the background measurement from either of the reference tissues indicating that the hyphae detected and morphologically described by microscopy were *R. solani*. Each micro-titer well in the ELISA contained the equivalent of 75 µg of tuber tissue. Bars = SE of the mean.

The dense growth of hyphae within this sample of PE tissue was made more visible when stained with trypan blue (Figure 6A). The hyphae branched at predominantly right angles. These right angle branches were somewhat constricted at the point of union to the progenitor hyphae. The hyphae were approximately 3 to 6 µm in diameter (Figure 6A). The morphological characteristics of the hyphae found within these cells are comparable with that of *R. solani* cultured *in vitro*, which were approximately 7 to 11 µm in diameter (Figure 6B).

ELISA Identification of Hyphae in PE Sample

The hyphae found in this PE tuber tissue were immunologically examined via ELISA using rabbit anti-*R. solani* antibody (Figure 7). Results show that the PE cortical parenchyma tissues, which were microscopically determined to harbor hyphae, bound about 33% more anti-*R. solani* antibody than the PE tissues and control tissues that did not show microscopical evidence of hyphae or *R. solani* sclerotia. These data indicate that the hyphal infections in these PE cortical parenchyma tissues were *R. solani* (Figures 5, 6, and 7).

DISCUSSION

The cellular profiles from this research clearly demonstrate that the insoluble bright subsurface autofluorescence that is characteristic of the presence of PE is derived from the large accumulation of autofluorescent poly(phenolic) material on the cell walls of the cortical parenchyma. Our results, based on cytological and histochemical characteristics, identify the autofluorescent material as SPP because (1) it accumulates and fluoresces in the same fashion as that found on suberizing closing layers during wound-healing, (2) SPA may subsequently accumulate over the material thereby defining the barrier as suberized, and (3) an internal phellogen may form under the closing layer and produce an internal periderm with files of phellem cells that block advancement of fungal hyphae.

The surfaces of mature PE tubers were characterized by areas where the native phellem had physicochemically deteriorated, or the phellem had delaminated from the tuber at the phellogen. Although in many cases the phellem appeared to be present and intact, subtle evidence obtained through microscopic examination frequently indicated that phellogen damage was present. In other cases there was a complete absence of phellem and associated suberin protection. Phellogen deterioration *in situ*, without obvious physical evidence, during tuber bulking would be extremely difficult to detect, but like excoriation, it would elicit development of a closing layer within the cortical parenchyma (Lulai and Morgan 1992; Lulai and Freeman 2001). PE-induced deterioration or absence of the native phellem is generally not obvious to the naked eye.

Lack of a suberin barrier on the surface of PE tubers, a stress damaged or killed phellogen, and/or a native periderm having a suberin barrier with compromised integrity likely contributed to the widespread induction of SPP accumulation on the cortical parenchyma cell walls. The PE-related suberization has similarities to wound-healing. However, unlike the typical wound-induced closing layer that is formed at the wound surface, PE induces widespread accumulation of SPP on cell walls deep within the cortical parenchyma of the tuber. SPA accumulation generally occurs excessively late or not at all on these existing cells so that the process of SPP accumulation continues unabated on neighboring cell walls. In some cases an internal phellogen developed. As with the development of a wound periderm, the PE-induced formation of an internal layer of meristematic cells (phellogen) terminated further accumulation of SPP on existing cell walls. At times, this

process resulted in the formation of an internal periderm consisting of files of suberized phellem cells, a phellogen and a putative phelloderm.

The saturated soil conditions, higher than normal temperatures, poor vine health, and associated canopy deterioration that predispose tubers to PE development could contribute to development of hypoxia and other conditions around the tuber tissues. Suberization is inhibited by temperatures approaching and exceeding 35 C (Wigginton 1974). The meristematic activity and viability of the phellogen of the native periderm and subsequent associated processes could also be adversely affected by these conditions and contribute to the PE syndrome described here. It is important to note that hypoxic conditions have been shown to result in the death of meristematic cells in potato root tissues (Biemelt et al. 1999). Diffusivity of oxygen in water is approximately 10,000 times slower than in air (Colmer 2003). Likewise waterlogged soils impede oxygen diffusion. Higher temperatures increase respiration, oxygen demand, and carbon dioxide production. These environmental conditions may also alter pH, favor growth of certain pathogens and collectively produce a variety of phytotoxic byproducts. The above conditions and other unknown factors could increase stress on the neighboring tuber periderm and play a role in PE development and associated infections.

PE also affected tuber water loss. Tuber water vapor conductance is controlled by soluble waxes that are embedded in the suberin matrix (Soliday et al. 1979; Lulai and Orr 1994; Schreiber et al. 2005). The aberrant increases of water vapor loss through PE tissues were consistent with the typical irregular deterioration and at times total absence of a suberized barrier. The wide "range" and associated SE for water vapor loss in PE tubers is also indicative of the large variation in PE severity and expression on each tuber surface and from tuber to tuber. Further statistical analysis of these measurements, beyond indications of variability, has little meaning because of this potential variation from apparent normalcy to detectable characteristics of the PE syndrome in single tubers. These vapor conductance results provide further evidence from an analysis distinctly different from the cytological approach that chemistries associated with suberin, but not detectable by microscopy, are lacking or altered and that the native periderm of PE surfaces is compromised. The high ranges in water vapor conductance through these PE areas may also play a role in the development of surface defects that are characteristic of PE as well as lead to shrinkage in tubers.

The integration of results on the presence and blockage of hyphae in PE tubers with the cytological results provides a more comprehensive understanding and characterization of the PE syndrome. Absence or deterioration of suberized cells in the native periderm of PE tubers rendered the tissues without an effective barrier to infection. The PE-induced accumulation of SPP on cortical parenchyma cell walls could protect the tuber from bacterial infections, but the lack of SPA would not provide a barrier to fungal infection. Those areas of the tuber that were initially afflicted with PE, such as the bud end, would be most vulnerable to infection since a fungal pathogen may readily breach the SPP barrier. Fungal infection could thereby broaden the infection court by providing an opening through the SPP barrier for secondary infection by bacteria. This process for fungal infection is consistent with the susceptibility hierarchy determined by Lulai and Corsini (1998). A lack of the SPA barrier in PE tubers explains the presence of fungal hyphae within the tissues of some samples. The morphology of the hyphae found within the tuber cells and the absence of incipient fungal rot were consistent with *R. solani* (Sneh et al. 1994; Banville et al. 1996; Keijer 1996). The presence of *R. solani* was confirmed by immunodetection. Although *R. solani* hyphae were present in the tuber cortical parenchyma, it was not established whether this is a saprophytic, facultative parasitic, or other relationship. However, because of the PE disorder, this fungus was able to penetrate the compromised tuber barriers and invade deeply into cortical tissues, thereby providing possible access to rot-type bacterial pathogens. Therefore, this fungal presence is referred to as an infection for the purposes of the remaining discussion. The potential for fungal infection, particularly by a non-rot-type pathogen such as *R. solani*, through the surface of PE-afflicted tubers further explains the variation in latent infections that are characteristic of this disorder. We emphasize that most of the samples analyzed did not have hyphae. The results provide unusual and vivid illustrations that this fungus, which is nearly always restricted to the surface of healthy tubers and is known to have irregular propagation/infection patterns (Banville et al. 1996), may infect the parenchyma of tubers with PE-compromised periderm. The presence of hyphae within the parenchyma clearly validates our cytological findings and interpretations regarding the roles of absent or weakened suberin barriers in PE infections. Other infection combinations may result in latent development of rots while more aggressive pathogens would cause rot in the

field and during early periods of storage. As found with many PE samples, tubers may develop PE, but do not always develop microbial colonizations/infections and associated rot. The absence of *R. solani* in most samples further indicates that *R. solani* is not required for PE, but penetration by this and other fungi would increase the susceptibility to bacterial infections and rot. Importantly, these results provide a plausible explanation and unusual illustration for the mechanism of PE infection court and rot anomalies and the non-causal association of various pathogens with PE.

In conjunction with the PE suberization data, it is important to note that although signals from *Verticillium* spp. have been shown to induce suberization of existing cells in the absence of a wound (Lulai 2005), Goth et al. (1993, 1994) determined that *Verticillium* spp. could exacerbate PE symptoms, but that it was not required for PE.

The use of neutral red as a stain for hyphae vacuoles in white light microscopy is well described (Weber 2002). However, this is the first known report showing that our combined toluidine blue O and neutral red treatment yields sensitive fluorescent detection of hydrophobic areas (vacuoles/lipid bodies) of hyphae with color contrast for non-fluorescent areas.

Additional research is needed to further characterize the physiology of the PE disorder so that rational approaches may be developed to determine the causal mechanism and means of control. These results indicate that care must be exercised in planning and interpreting PE-related research because the environmental stresses and conditions that are associated with PE development may also induce other physiological disorders, such as heat necrosis, as well as exacerbate infections by a variety of pathogens that may be present. These disorders and infections may not be part of the PE complex.

In summary, this research creates a new physiologically based model for PE that explains why Koch's postulates could not be completed with any of the pathogens tested to date including those sporadically associated with this disorder. The results establish that in PE tubers there is a deterioration of the structural integrity of the native periderm that results in susceptibility to infection by a variety of pathogens. This research identifies the source of PE autofluorescence and clearly shows that a compromised suberin barrier and aberrant suberization are characteristic of the disorder and subsequent disease issues.

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